Therapeutic Potential of Small Molecules and Engineered Proteins

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Abstract

Virtually all currently used therapeutic agents are small molecules, largely because the development and delivery of small molecule drugs is relatively straightforward. Small molecules have serious limitations: drugs of this type can be fairly good enzyme inhibitors, receptor ligands, or allosteric modulators. However, most cellular functions are mediated by protein interactions with other proteins, and targeting protein–protein interactions by small molecules presents challenges that are unlikely to be overcome with these compounds as the only tools. Recent advances in gene delivery techniques and characterization of cell type-specific promoters open the prospect of using reengineered signaling-biased proteins as next-generation therapeutics. The first steps in targeted engineering of proteins with desired functional characteristics look very promising. As quintessential scaffolds that act strictly via interactions with other proteins in the cell, arrestins represent a perfect model for the development of these novel therapeutic agents with enormous potential: custom-designed signaling proteins will allow us to tell the cell what to do and when to do it in a way it cannot disobey.

1 Direct Action Drugs: Ligands and Inhibitors

Virtually all traditional drugs, as well as >90 % of therapeutics currently marketed are small molecules (Hopkins and Groom 2002). To be clinically useful, a drug has to be fairly selective, which means that it must bind its target with relatively high affinity, with K_D in the nanomolar range or better. There is direct relationship between the energy of the interaction and the K_D: $G^0 = -RT\ln(1/K_D)$, where G^0 is change in free energy due to interaction, R is gas constant (1.99 cal/mol degree), and T is temperature (in degrees Kelvin). By virtue of its size a small molecule has few chemical moieties that can engage its target. With very few exceptions reasonably high affinity is only achieved when the target protein "envelopes" the drug, i.e., only cavities or deep grooves in any protein can be

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successfully targeted. This determines severe thermodynamic limitations to what a small molecule can do. Indeed, among targets of marketed drugs about half are enzymes with a deep catalytic cleft where the drug binds, and most of the rest are receptors, ion channels, or transporters equipped with deep cavities where drugs interact (Hopkins and Groom 2002). Since any nonsubstrate that binds at the catalytic cleft of an enzyme acts as an inhibitor, virtually all enzyme-targeting drugs are inhibitors (Imming et al. 2006). The same is true for transporters. Receptors offer wider range of possibilities despite these limitations: a drug binding in the same cavity where natural ligand binds can be an activator (agonist), neutral antagonist, or an inverse agonist suppressing constitutive activity of the receptor, all competing for the same binding site (Imming et al. 2006). Drugs with all of these modalities are used, well-known examples being beta-blockers (antagonists) used to treat heart diseases and beta-agonists used in asthma.

The key drawbacks of conventional small molecule therapeutics are that they are essentially "one-trick ponies" that can do only one thing and that they keep doing it regardless of the physiological state of the patient, because they are not equipped to receive feedback from the body. For example, if you take a beta-blocker for your heart condition, it will keep blocking beta-adrenergic receptors when you are sitting and using relatively little energy and therefore needing fairly slow heart rate, as well as when you are running and using a lot more energy and oxygen, which requires harder work from the heart to provide increased blood flow. In addition, beta-blocker will block beta-adrenergic receptors in other tissues, which can cause side effects. That is why most drugs come with numerous warnings telling you what to do and not to do after taking the drug, describing various possible side effects, and advising you to stop taking the drug if these unwanted effects are too strong.

There is one area where small molecules are and will likely remain the best possible therapeutic tools: fighting parasites, such as bacteria, fungi, and viruses. Small molecule inhibitors are very effective as antimicrobials because they target enzymes performing biochemical reactions that we don't have, such as building and maintaining cell wall. The most effective antibiotics inhibit enzymes involved in cell wall construction (e.g., penicillin and the whole family of its derivatives) or something else specific for the bacteria, like their ribosomes that are very different from eukaryotic ones. RNA viruses can be selectively targeted via their reverse transcriptases, as our only enzyme in this class, telomerase, is quite different, and so on. However, when our own proteins need to be regulated for therapeutic purposes, "single-mindedness" of enzyme inhibitors or receptor ligands, as well as their unresponsiveness to the signals sent by the rest of the body becomes a huge liability.

2 Allosteric Modulators: Greater Sophistication

Small molecules have several obvious advantages. First, new small molecule drugs targeting the enzyme or receptor of interest can be devised using well-established procedures (Segall 2012). New compounds with therapeutic potential can be created by generation of new derivatives of known compounds and then selection of the most potent and specific among them. Alternatively, completely new compounds targeting a particular protein can be identified by high-throughput screening of widely available huge chemical libraries and then the same process of generation of derivatives and selection can be applied (Mayr and

Bojanic 2009). Both approaches are conceptually straightforward, although quite expensive. Second, it is also fairly well known which chemical groups in putative drugs should be avoided to prevent poor absorption in the gut or rapid metabolism, so this part of drug development also does not require any intellectual breakthroughs, only more funding. Therefore, it was natural that the first attempts to overcome some of the limitations of conventional drugs focused on small molecules.

Receptors are usually medium-sized proteins, where endogenous agonists and conventional orthosteric ligands, all interacting with the same site, that are used as drugs occupy only a small area. The binding of compounds to other parts of the receptor can enhance or reduce activating effect of the agonist, thereby modulating the signaling (Luttrell and Kenakin 2011). To a certain extent, the development of positive and negative allosteric modulators of G protein-coupled receptors (GPCRs) solved one key problem of small molecules. Modulators only act in conjunction with endogenous agonists, decreasing or increasing their action, but remain essentially inactive in its absence (Kenakin and Miller 2010). Thus, the effect of modulators depends on the physiological state of the patient, which makes them superior to conventional orthosteric ligands. This explains rapid expansion of research in this area.

However, even allosteric modulators share quite a few limitations of conventional drugs. First, each of these molecules is designed to do only one thing: it targets an individual receptor (and is carefully selected for this narrow specificity). Second, the only feedback the allosteric modulators respond to is the level of endogenous orthosteric ligand. In addition, the strongest positive modulators at higher concentrations act as allosteric agonists, stimulating the signaling even in the absence of endogenous ligands (Kenakin 2010). Thus, considering the complexity of biological systems, the limited set of functional capabilities of any small molecule remains an unavoidable disadvantage of this approach.

3 Protein-Based Therapeutics: Challenges and Potential

It is widely known that virtually all vital aspects of cellular behavior, such as adhesion, migration, proliferation, and cell death by apoptosis or other mechanisms, are mediated and regulated via interactions of proteins with each other (Elowitz and Lim 2010). Most extracellular signals exert their action by promoting or disrupting interactions of particular proteins in the cell. For example, in case of GPCRs, which are targeted by >30 % of clinically used drugs (Hopkins and Groom 2002), the agonists promote receptor interactions with heterotrimeric G proteins (Samama et al. 1993), then GPCR kinases (GRKs) (Gurevich et al. 2012), and then arrestins (Gurevich and Gurevich 2006). Receptor-dependent activation of G proteins induces dissociation of their α- and βγ-subunits, promoting their interactions with various effector proteins (Dessauer et al. 1996). Receptor-bound arrestins bind clathrin and AP2 (Goodman et al. 1996; Laporte et al. 1999), which triggers GPCR internalization via coated pits, and interact with numerous other proteins, initiating the second round of signaling (Hanson et al. 2006; Xiao et al. 2007). Chains of sequential protein–protein interactions underlie every signaling pathway in the cell.

Therefore, the ability to selectively disrupt or enhance individual protein–protein interactions would give us an unprecedented leverage over the cell, essentially allowing us to tell the cell what to do and when to do it in a language it understands (Gurevich and Gurevich 2012). This will be hugely advantageous scientifically, giving us powerful tools to elucidate the intricacies of cell signaling, which is arguably the greatest current challenge in biological research. This will also pave the way to devising conceptually novel therapeutic approaches with potential to actually cure many congenital and acquired diseases, in contrast to just managing the symptoms, which is the best we can do now in case of asthma, diabetes, depression, mental disorders, heart disease, Parkinson's, Alzheimer's, retinal degeneration, etc.

However, protein–protein interactions are virtually never targeted for therapeutic purposes. Naturally, this is not an oversight: there are real difficulties in targeting these interactions with small molecules, which currently predominate as therapeutic tools. First, protein elements mediating the interaction are very rarely mapped with necessary precision to be targeted, or in most cases are simply unknown (Gurevich and Gurevich 2010). Second, the elements involved are often unstructured ("intrinsically disordered"), and only assume final fold upon interaction, with the help of the binding partner. This coupled folding and binding is sometimes referred to as "fly-casting mechanism" (Shoemaker et al. 2000; Sugase et al. 2007). It is currently impossible to design a small molecule targeting a disordered polypeptide. Most importantly, even when the interacting elements are identified and well ordered with known three-dimensional structure, the interactions are mediated by relatively flat protein surfaces, which do not bind small molecules with high enough affinity (in contrast to deep grooves, like the active sites of most enzymes or ligand-binding sites of receptors). These surfaces are usually also too large [$>2,000 \text{ Å}^2$ (Jones and Thornton 1995)] to be significantly modified by a small molecule. These structural limitations suggest that it is highly unlikely that small molecules selectively targeting most individual protein–protein interactions will ever be developed. Last, but not least, every intervention with small molecules attempted so far aimed at disrupting protein–protein interactions (Thiel et al. 2012), whereas it is equally likely that selective strengthening of some of them will be of high scientific and therapeutic value.

The most realistic way of modulating protein–protein interactions in a desired manner is to rely on proteins themselves. By introducing into the cell a protein with modified signaling properties we can affect cell behavior as we like. Cancer cells represent one obvious target: if we could tell them to stop proliferating, that would solve the problem. Another obvious target is dying neurons in neurodegenerative diseases: if we could tell them to stay alive in a way they cannot disobey, we would have a cure. Biological function of signaling proteins is to deliver messages. Thus, we need to learn how to create our own messengers to deliver signals we want and/or to override the signals we disagree with that the cell receives from other sources.

Gene delivery to targeted cell types in humans is no longer science fiction. The development of viral and nonviral gene delivery systems (Bartel et al. 2012; Nguyen and Szoka 2012) and identification of promoters driving the expression in cell types of choice is proceeding at a rapid pace. Recent success of three gene therapy clinical trials where correct RPE65 gene

was delivered to the pigment epithelium of Leber's congenital amaurosis patients carrying loss-of-function mutations in this protein (Cideciyan et al. 2008; Hauswirth et al. 2008; Maguire et al. 2008; Bainbridge et al. 2008) demonstrate that gene delivery methods are ready for use today, not in the distant future (Cideciyan 2010; Cao et al. 2011). The elucidation of fine molecular mechanisms of the function of every signaling protein would allow us to design signaling-biased mutants worth delivering by these sophisticated methods. It is particularly important to elucidate general principles of protein–protein interactions and the functional connections within cellular signaling networks to construct custom-designed signaling proteins with the functional characteristics we want and other protein-based molecular tools to tell cells what to do in a way they cannot ignore.

4 Signaling-Biased Arrestins as a Model

In most cases what we need to do for therapeutic purposes is selectively enhance or reduce only one interaction of a particular signaling protein out of a dozen or more it is normally engaged in. Arrestin proteins appear to be a perfect model to test-drive this approach for several reasons. First, arrestins are not enzymes or receptors with a binding pocket that can be targeted by small molecules. Arrestins are classical signaling scaffolds: everything they do in the cell is mediated by their interactions with other proteins (Gurevich and Gurevich 2003; DeWire et al. 2007). That is why currently there are no ways to affect their functions by small molecules, and it is highly unlikely that any drugs suitable for this purpose will ever be developed. Second, mammals express only two nonvisual arrestins, arrestin-2 (a.k.a. β-arrestin1)[1] and arrestin-3 (a.k.a. β-arrestin2), each interacting with hundreds of different G protein-coupled receptors (GPCRs) and dozens of other signaling proteins (Hanson et al. 2006; Xiao et al. 2007). Third, arrestins are ubiquitous signaling regulators in the cell, involved in multiple pathways, including several that directly regulate cell fate via pro-survival or pro-apoptotic signaling (Gurevich and Gurevich 2010, 2012). This makes arrestins convenient ubiquitously expressed tools for modulating cell behavior.

Structurally, arrestins are characterized better than most signaling scaffolds. Crystal structures of all four vertebrate arrestins have been solved (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011), as well as the structures of arrestin-2 complexes with the interacting elements of clathrin (ter Haar et al. 2000; Kang et al. 2009). Moreover, extensive structure–function studies of arrestin family members revealed the function of numerous amino acid side chains in these relatively small 40–45 kDa proteins [reviewed in Gurevich and Gurevich (2004, 2006) and Gurevich et al. (2011)]. The dynamics of arrestin molecule in solution was studied by a variety of methods, including H/D exchange (Ohguro et al. 1994; Carter et al. 2005), site-directed spin labeling and electronic paramagnetic resonance (EPR) (Hanson et al. 2006, 2007a, b, 2008; Vishnivetskiy et al. 2010, 2011), and nuclear magnetic resonance (NMR) with $^{13}C/^{15}N$ labeled arrestin (Zhuang et al. 2010, 2013). Even though the structure of the arrestin–- receptor complex still remains to be solved, receptor binding-induced conformational changes in arrestins were recently

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[1]Different systems of arrestin names are used in the field and in this book. We use systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called "*arrestin 3*" in the HUGO database).

characterized by intramolecular distance measurements using double electron–electron resonance (Kim et al. 2012). The same mechanism of arrestin activation by receptor-attached phosphates was shown to operate in all arrestins (Gurevich and Benovic 1995, 1997; Gurevich 1998; Kovoor et al. 1999; Vishnivetskiy et al. 1999; Celver et al. 2002; Sutton et al. 2005). This uniformity was further supported by recently solved structures of the arrestin-2 with C-terminal deletion (Kovoor et al. 1999) in complex with multi-phosphorylated peptide representing the C terminus of the V2 vasopressin receptor (Shukla et al. 2013) and of similarly truncated short splice variant of arrestin-1, p44 (Kim et al. 2013). Both structures, which indicate likely direction of the receptor binding-induced conformational changes, representing arrestins somewhere between basal and receptor-bound state, turned out to be remarkably similar (Kim et al. 2013; Shukla et al. 2013).

The feasibility of structure-based redesign of arrestins to generate mutants with functional characteristics changed in desired direction has already been demonstrated. Based on the mechanism of arrestin activation by receptor-attached phosphates, the first signaling-biased arrestin mutants that bind with high affinity active unphosphorylated GPCRs were constructed (Gurevich and Benovic 1995, 1997; Gurevich et al. 1997; Gurevich 1998; Kovoor et al. 1999; Celver et al. 2002; Vishnivetskiy et al. 2013a, b). These enhanced arrestins were shown to quench signaling by unphosphorylated receptors in biochemical experiments with purified proteins in vitro (Gray-Keller et al. 1997), in intact cells (Kovoor et al. 1999; Celver et al. 2002), and in transgenic animals in vivo (Song et al. 2009). Enhanced arrestin mutants and their therapeutic potential are discussed in Chap. 7. Receptor-binding surface of arrestins was mapped by several groups, all of which identified multiple residues on the concave side of both arrestin domains as the receptor "footprint." The agreement on this point is rather remarkable, considering wide variety of methods used: H/D exchange (Ohguro et al. 1994), peptide competition (Pulvermuller et al. 2000), element swapping (Gurevich et al. 1993, 1995; Vishnivetskiy et al. 2004), epitope insertion (Dinculescu et al. 2002), site-directed mutagenesis (Hanson and Gurevich 2006; Vishnivetskiy et al. 2011), site-directed spin labeling/EPR (Hanson et al. 2006; Vishnivetskiy et al. 2010, 2011; Kim et al. 2012), and NMR (Zhuang et al. 2013). The finding that very few residues on this extensive surface largely determine receptor specificity (Vishnivetskiy et al. 2011) was unexpected, but entirely welcome. The very first attempt of targeted mutagenesis of identified receptor-discriminator residues yielded versions of inherently promiscuous arrestin-3 with >50-fold preference for some GPCRs over others (Gimenez et al. 2012). The prospects of constructing arrestins specifically targeting groups of receptors or even individual GPCRs are discussed in Chap. 8.

We are approaching the limits of what can be achieved in a complex living organism with small molecules, suggesting that more sophisticated tools are needed. Custom-designed signaling proteins with special functional characteristics are the "smarter" tools we need that along with regulatory RNAs (that also require gene delivery) will likely become next-generation therapeutics. Using reengineered proteins we can manipulate cell signaling in ways that cannot be achieved by other means. Targeted mutations change protein–protein interactions that due to their structural properties most likely will never be successfully targeted by small molecules. In contrast to small molecules that have a single function and do not respond to the physiological state of the patient, proteins with targeted modifications

will remain sensitive to normal feedback mechanisms operating in the cell. This minimizes the chances of severe adverse side effects, which arguably doomed more conventional drugs than any other issue.

Critical roles that arrestin proteins play in many biological processes make them a perfect target to develop and test new approaches of manipulating cell signaling for research and therapeutic purposes. Numerous arrestin functions and their structural basis are discussed in this book. These include the mechanisms of receptor binding (Chap. 2), the action of arrestin-biased GPCR agonists (Chap. 3), as well as specific functions of visual subtypes (Chaps. 4–6), the possibility of compensating for the lack of receptor phosphorylation with enhanced arrestins (Chap. 7) and creating mutant forms of nonvisual arrestins to target specific GPCRs (Chap. 8). In addition to hundreds of GPCRs, arrestins interact with a variety of other proteins (Gurevich and Gurevich 2006; DeWire et al. 2007). Identification of arrestin elements engaging non-receptor partners enabled the construction of mutants where one particular function was disabled, leaving the others virtually intact (Kim and Benovic 2002; Meng et al. 2009; Coffa et al. 2011; Kim et al. 2011; Seo et al. 2011; Breitman et al. 2012). The mechanisms of clathrin and AP2 binding and properties of arrestins lacking these functional modalities are discussed in Chap. 9. The role of arrestins in protein ubiquitination and deunbiquitination are discussed in Chap. 10. The elements involved in self-association of visual and nonvisual arrestins and characteristics of constitutively monomeric mutants are discussed in Chap. 11. Several chapters discuss the role of arrestins in the activation of MAP kinases ERK1/2 (Chap. 12), JNK1/2/3 (Chap. 13), and p38 (Chap. 14). This book also discusses arrestin roles in a variety of other biological processes, such as regulation and localization of PDE (Chap. 15), programmed cell death (Chap. 16), cell motility (Chap. 17), infectious diseases and host–pathogen interactions (Chap. 18), regulation of small GTPases (Chap. 19), airway epithelium and asthma (Chap. 20), cancer (Chap. 21), as well as pain and anesthesia (Chap. 22). Despite enormous breadth, even this volume is not exactly comprehensive, but it gives the reader an idea of the variety of biological roles played by a small family of four vertebrate arrestins.

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